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Biogenesis and metabolic significance of microbodies in urate-utilizing yeasts

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Growth of *Candida famata* and *Trichosporon cutaneum* on uric acid as the sole source of carbon and nitrogen was associated with the development of a number of microbodies in the cells. Cytochemical staining experiments showed that the organelles contained urate oxidase, a key enzyme of uric acid metabolism, and catalase. Transfer of cells, precultured on glucose or glycerol, into uric acid-containing media indicated that these microbodies originated from the organelles, originally present in the inoculum cells, by growth and division. In urate-grown *C. famata* the microbodies were frequently observed in large clusters; in both organisms they existed in close association with mitochondria and strands of ER. The organelles lacked crystalline inclusions. In freeze-fractured cells their surrounding membranes showed smooth fracture faces.

Exposure of urate-grown cells to glucose-excess conditions led to a rapid inactivation of urate oxidase activity but catalase was only slightly inactivated. Glucose-induced enzyme inactivation was not associated with the degradation of the microbodies present in the cells. Similarly, repression of urate oxidase synthesis by ammonium ions also did not lead to the degradation of peroxisomes.

INTRODUCTION

The ability of yeasts to utilize uric acid as the sole source of nitrogen is well-known (LaRue and Spencer, 1968). Previous studies have demonstrated that as in microorganisms that can use uric acid as a carbon source (Vogels and Van der Drift, 1976), urate oxidase was the key enzyme involved in urate metabolism in yeasts. In the latter organism this enzyme is present in peroxisomes

which developed in small numbers during growth of various yeast species in media containing uric acid as a nitrogen source (Fukui and Tanaka, 1979; Veenhuis et al., 1983*b*; Veenhuis and Harder, 1985).

Recently a number of yeast strains have been isolated that are capable of growth on uric acid as the sole source of carbon and nitrogen (Middelhoven et al., 1983). These organisms were identified as strains of *Candida famata* and *Trichosporon cutaneum*. At the subcellular level growth of these organisms at the expense of uric acid was associated with the development of large numbers of microbodies in the cells. This latter finding prompted us to investigate the metabolic significance of the microbodies. Since the number of microbodies per cell, compared with cells grown on uric acid as the nitrogen source had considerably increased, we have also studied the influence of cultivation conditions on the degree of microbody proliferation. In addition, the mechanisms involved in microbody biogenesis and turnover have been investigated and compared with general mechanisms which have recently been postulated to be involved in the development and turnover of microbodies (peroxisomes/glyoxysomes) in the yeasts *Hansenula polymorpha* and *Candida utilis* under different environmental conditions (Veenhuis et al., 1983*b*; Zwart, 1983; Veenhuis and Harder, 1985). The results of these studies are presented in this paper.

Materials and Methods

Microorganism and cultivation

Candida famata CBS 8109 and *Trichosporon cutaneum* CBS 8110 were cultivated at 30°C in aerated Kluyver flask cultures containing 300 ml of the mineral medium described previously (Middelhoven et al., 1983), supplemented with 0.5% (w/v) glucose or 1.0% (v/v) glycerol as the carbon source and 0.25% (w/v) ammonium chloride as the nitrogen source. Cells from the mid-exponential growth phase were washed once in the mineral medium and transferred to shake flask cultures supplemented with either 0.4 or 0.5% (w/v) uric acid as the combined carbon and nitrogen source, 0.4 or 0.5% (w/v) uric acid plus 0.25% (w/v) ammonium sulphate, or 0.3% (w/v) glucose plus 0.1% (w/v) uric acid. For enzyme assays a more buffered mineral growth medium (20 g $KH_2PO_4 \cdot l^{-1}$ instead of 1 g $\cdot l^{-1}$) was used. It was supplemented with 5 g glucose per litre (*T. cutaneum*) or 10 g glycerol per litre (*C. famata*). The nitrogen source was 2 g ammonium chloride per litre. Cultivation of cells and adaptation to uric acid of washed cells took place in a vigorously aerated Kluyver flask at 30°C.

Transfer experiments

Cultures of *C. famata* and *T. cutaneum* in the exponential growth phase on urate as the sole carbon and nitrogen source (judged by the degree of disappearance of uric acid crystals) were supplemented with 0.3% (w/v) glucose and/or

0.25% (w/v) ammonium sulphate. In addition, stationary phase cells were transferred into fresh media with glucose and ammonium sulphate. As a control cells were transferred into media lacking the carbon and the nitrogen source. Samples were taken at regular time intervals.

Enzyme assays

Cell-free extracts were prepared by sonication of whole cells. Protein and allantoinase were assayed as described previously (Middelhoven, 1977). Catalase was determined spectrophotometrically at 240 nm by following the enzyme-catalysed decomposition of H_2O_2 (4 mM) in 0.1 M potassium phosphate buffer at pH 7.0. Urate oxidase was determined spectrophotometrically by following the decrease of the absorbance at 293 nm in an assay mixture containing 0.1 mM uric acid in 0.1 M Tris-HCl buffer pH 9.0. Enzyme assays were performed at 30°C, and the specific activities are expressed as μmol substrate converted $\cdot$mg protein $^{-1} \cdot h^{-1}$.

Cytochemical staining techniques

The cytochemical staining experiments were performed on glutaraldehyde-fixed cells. Catalase activity was demonstrated with diaminobenzidine (DAB) and H_2O_2 (Veenhuis et al., 1976). Urate oxidase was demonstrated using the $CeCl_3$-technique according to the methods described previously (Veenhuis et al., 1976).

Freeze-etching

Cells were incubated in 10% (v/v) glycerol for 2–5 min, frozen in Freon and freeze-fractured in a Balzer's freeze-etch unit according to the method described by Moor (1964).

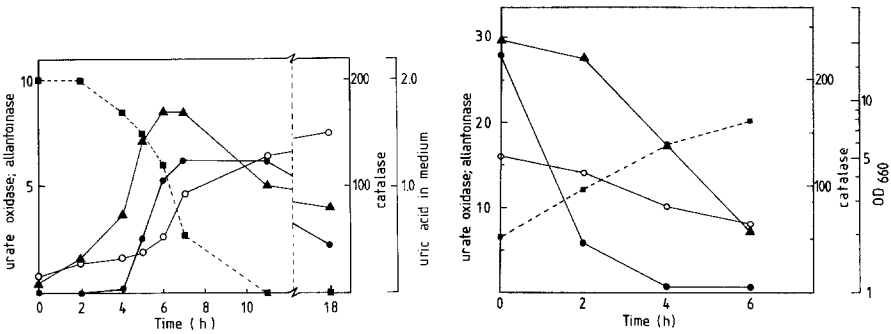
Fixation and post-fixation techniques

Whole cells – also after cytochemical staining techniques – were fixed with 1.5% (w/v) $KMnO_4$ for 20 min at room temperature. After dehydration in a graded ethanol series, the material was embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM300 without further staining. The number and volume fraction of peroxisomes was determined on thin sections by the method described previously (Veenhuis et al., 1979).

Results

Biochemical experiments

After transfer of glucose- or glycerol-grown cells of *C. famata* or *T. cutaneum* into media containing uric acid as the combined carbon and nitrogen source,



Figs 1, 2. Growth, uric acid consumption and enzyme profiles in batch cultures of *Trichosporon cutaneum* (Fig. 1) and *Candida famata* (Fig. 2) after transfer of cells from the exponential growth phase on glycerol (Fig. 1) or glucose (Fig. 2) into media supplemented with uric acid as the sole source of carbon and nitrogen. ■–■, uric acid in culture medium (g per 500 ml); ●–●, urate oxidase; ▲–▲, catalase; ○–○, allantoinase.

the consumption of uric acid started after a lag of approximately 2 h and was associated with a rapid increase in catalase, urate oxidase and allantoinase activity in the culture (Figs 1, 2). Since uric acid is a sparingly soluble substrate, the change in biomass was difficult to assess. Therefore, the decrease in the amount of uric acid present in the culture vessel was taken as a measure for growth. Judged by this criterion catalase and urate oxidase reached their maximum activities at the end of growth and remained rather stable thereupon (Figs 1, 2). Similarly, in both strains allantoinase activity reached its maximum value in the stationary growth phase.

Transfer of uric acid-grown *C. famata* cells taken from the early stationary growth phase into media containing glucose and ammonium sulphate resulted in a rapid decrease in urate oxidase activity (Fig. 3). Catalase activity only slightly decreased after 2 h of cultivation, whereas the observed decrease in allantoinase activity could be accounted for by dilution of enzyme protein as a result of growth. Essentially similar results were obtained when glucose was added to exponentially growing cultures of *C. famata* growing on uric acid as the sole C- and N-source. Control experiments revealed that the observed decrease in urate oxidase was dependent upon the presence of glucose in the media. Transfer of cells into mineral media without any carbon or nitrogen source or media with ammonium sulphate as the N-source but lacking a carbon source did not result in inactivation of catalase, urate oxidase or allantoinase. In these experiments the observed decrease in enzyme activity could be explained by dilution of enzyme protein as a result of growth. Similar patterns of enzyme inactivation

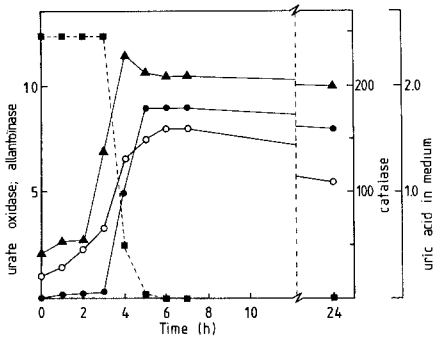


Fig. 3. Growth and enzyme profiles in batch cultures of *Candida famata* after transfer of cells from the early stationary growth phase on uric acid into media supplemented with glucose and ammonium sulphate. ■–■, growth, expressed as optical density of the culture at 660 nm. The other symbols are as in Fig. 1.

were obtained when *T. cutaneum* was transferred from uric acid- into glucose-containing media.

Electron microscopy

Cells of *T. cutaneum* from the exponential growth phase on glucose contained a few small microbodies which were irregular in shape and measured up to 0.2 μm (Fig. 4). They were partly located in the proximity of the cell wall and partly closely associated with the nucleus; in the latter case they showed a typical bean-like shape (cf. inset Fig. 4). Transfer of such cells into media containing uric acid as the sole carbon and nitrogen source led to a rapid increase in size of these organelles (Fig. 5). This process was already initiated in the lag phase which occurred during adaptation of cells to urate metabolism. During the subsequent growth on uric acid the number of microbodies per cell gradually increased by the separation of small organelles from mature ones, thus resulting in the presence of large numbers of microbodies in cells from the stationary growth phase (Fig. 6). Up to sixteen microbody profiles have been observed in thin sections of $KMnO_4$-fixed cells; the organelles were highly irregular in shape with dimensions up to 0.9 μm. Although in urate-grown *T. cutaneum* the microbodies were generally contained in one part of the cell, they predominantly existed as separate organelles; generally, no clusters of peroxisomes were observed (cf. Fig. 6). Similar results were obtained when ethanol-grown cells were used as an inoculum for urate-containing media. Also in these experiments the microbodies which developed in the urate-grown cells originated from those already present in the inoculum cells by growth and division. Similarly, also the microbodies present in developing buds during vegetative reproduction of cells on urate were

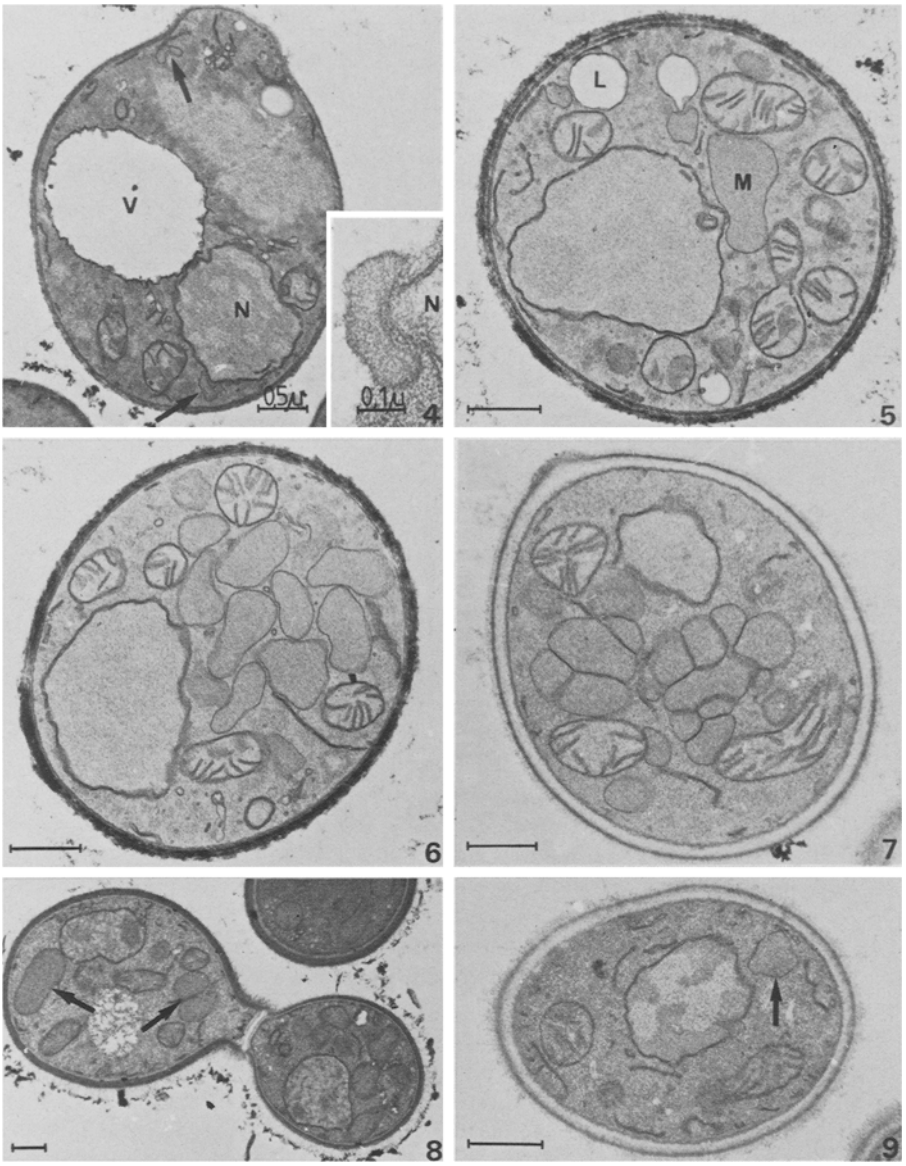
not synthesized 'de novo' but originated from the organelles present in the mother cell.

The mechanisms, described above for the development of microbodies in *T. cutaneum* during adaptation and subsequent growth on uric acid, were also operative in *C. famata* when grown under identical conditions. The only difference observed was that the microbodies in *C. famata* invariably were present in large clusters, consisting of up to twelve microbodies in cells taken from the stationary phase of growth (Fig. 7).

The results of the biochemical experiments (Fig. 3) indicated that the synthesis of urate oxidase is partially repressed by glucose and ammonium ions. This is in agreement with the results of morphometrical analysis, which revealed that the number and volume density of the microbodies during growth of cells on urate were largely influenced by the composition of the growth medium. Maximum values were observed under conditions in which uric acid served as the combined carbon- and nitrogen source; smaller numbers were present in cells grown on uric acid in the presence of ammonium sulphate as the nitrogen source whereas the volume density of the microbodies was lowest when uric acid served as the sole nitrogen source in media containing glucose as the carbon source (Figs 8,9; Table 1). The microbodies present in both organisms studied had a number of properties in common: they were frequently closely associated with the mitochondria and strands of endoplasmic reticulum, lacked crystalline inclusions whereas in freeze-etch preparations, their surrounding membranes showed smooth fracture faces (Fig. 10) similar to those described previously for *H. polymorpha* and *C. utilis* (Veenhuis et al., 1983*b*; Zwart, 1983).

Cytochemical staining experiments indicated that the observed increase in microbody size during growth of *T. cutaneum* or *C. famata* on uric acid was most probably due to the import of urate oxidase protein. Already in the early stages of growth on urate – 4 h after the transfer of cells – urate oxidase activity could be demonstrated cytochemically in the different microbodies present in the cells (Fig. 11), irrespective whether glucose- or ethanol-grown cells were used as the inoculum. In addition to urate oxidase also catalase activity was demonstrated in these organelles and therefore accumulation of this enzyme may add to the observed increase in microbody volume density. The cytochemical experiments indicated that, judged by the localization of the reaction products, the activities of both urate oxidase and catalase were confined to the peroxisomal matrix (Fig. 12, 13). The additional staining of the mitochondria in the DAB-based incubations occurred irrespective of the presence of substrate, was inhibited by cyanide ions and therefore was most probably due to staining of mitochondrial peroxidases (Hoffmann et al., 1970; Van Dijken et al., 1975).

The rapid inactivation of urate oxidase in *T. cutaneum* and *C. famata* observed after exposure of urate-grown cells to glucose-excess conditions (cf. Fig. 3), was not associated with the degradation of the microbodies present in the cells. Similarly, also the presence of ammonium sulphate did not lead to the degradation of the microbodies.



Figs 4, 5. Thin sections of cells of *Trichosporon cutaneum* from the exponential growth phase on glucose, showing two microbody profiles (Fig. 4; arrows) and the increase in microbody size 6 h after transfer of cells to uric acid-containing media (Fig. 5). The inset of Fig. 4 shows a typical bean-like-shaped organelle, frequently observed in such cells in close association with the nucleus.
Figs 6–9. Micrographs showing the proliferation of microbodies in cells from the early stationary growth phase on uric acid as the sole C- and N-source (Figs 6, 7), uric acid/ammonium sulphate (Fig. 8) and glucose/uric acid (Fig. 9). In Figs 8 and 9 microbodies are indicated by arrows. Figs 6, 8: *Trichosporon cutaneum*; Figs 7, 9: *Candida famata*.
All electron micrographs are taken of $KMnO_4$-fixed cells; the marker represents 0.5 μm. Abbreviations: L, lipid droplet; M, microbody; N, nucleus; V, vacuole.

Table 1. The proliferation of microbodies in *Candida famata*, expressed as number and volume density of the organelles in relation to different conditions of growth[1]

Growth conditions		Number of peroxisomes	Volume density of peroxisomes
C-source	N-source		
Glucose	ammonium sulphate	0.1	0.2
Glucose	uric acid	0.3	1.8
Uric acid	ammonium sulphate	2.1	6.9
Uric acid	uric acid	4.2	10.1

[1] Cells were grown in shake flask cultures and harvested during the late exponential growth phase. The number of microbodies is expressed as average number per section, the volume density as percentage of the cytoplasmic volume.

Discussion

Growth of yeasts on uric acid as the sole source of carbon and nitrogen is associated with the presence of increased numbers of microbodies in the cells (Middelhoven et al., 1983). The results of our present study indicate that these organelles are the sole sites of urate oxidase activity (key enzyme in urate metabolism) in the cells and since they also contained catalase they must be considered as peroxisomes. In fact during growth of the yeasts on uric acid these organelles are involved in the initial metabolism of the combined carbon and nitrogen source and therefore all the carbon and nitrogen required for growth flows via peroxisomes. This property of peroxisomes in urate-grown yeasts is not unique since also during growth of *C. utilis* on D-alanine a peroxisomal enzyme, namely D-amino acid oxidase, is involved in the primary metabolism of the carbon and nitrogen source (Zwart et al., 1983*a*). In addition, several examples have been described in which two different peroxisomal oxidases are involved in carbon and nitrogen metabolism, for instance during growth of cells of *H. polymorpha* on methanol as the C-source and methylamine, D-alanine or uric acid as the N-source (Veenhuis et al., 1981, 1983*b*; Zwart, 1983). Our results clearly indicate that the peroxisomes present in urate-grown cells of *T. cutaneum* and *C. famata* develop, similarly as described for other yeasts (Veenhuis et al., 1983*b*; Veenhuis and Harder, 1985) from pre-existing organelles present in the cells prior to their transfer to the new environment. Also in these organisms this process is independent of the former metabolic function(s) of the organelles.

Thus, peroxisomes may develop from glyoxysomes; intermediate forms may also exist, for instance, during growth of cells on ethanol as the C-source and uric acid as the N-source. Therefore, similarly as described for *H. polymorpha* and *C. utilis* (Zwart et al., 1983*c*; Veenhuis and Harder, 1985) the microbodies

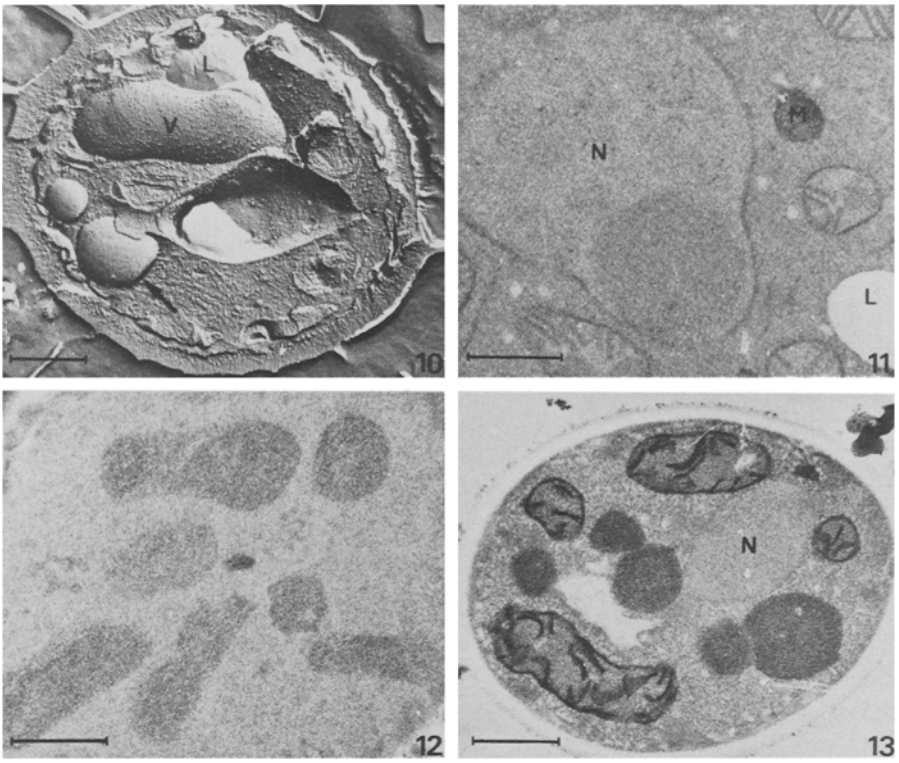


Fig. 10. Survey of a cell of *Candida famata*, grown on uric acid, showing the smooth fracture faces (arrow) of the microbody membrane after freeze-fracturing.
Figs 11–13. Cytochemical staining experiments showing the presence of urate oxidase in microbodies in *Trichosporon cutaneum*, 4 h after transfer of cells from glucose to uric acid-containing media (Fig. 11) and in cells of *Candida famata* from the stationary growth phase on uric acid (Fig. 12) together with catalase (Fig. 13). Urate oxidase was demonstrated with $CeCl_3$ and uric acid, catalase with DAB and H_2O_2.
All electron micrographs are taken of $KMnO_4$-fixed cells; the marker represents 0.5 μm. Abbreviations: L, lipid droplet; M, microbody; N, nucleus; V, vacuole.

in *T. cutaneum* and *C. famata* constitute one class of organelles; their ultimate physiological function(s) (peroxisomal, glyoxysomal or glyoxyperoxisomal in nature) is (are) largely prescribed by environmental conditions. The presence of a possible biosynthetic function, as recently described for microbodies in *H. polymorpha* and *C. utilis* (Zwart et al., 1983*b*) is yet unknown.

As in previous studies in other yeasts, the number and volume density of the microbodies in *T. cutaneum* and *C. famata* are largely dependent on the composition of the cultivation media. This is, for instance, illustrated by experiments in which cells were grown in media containing uric acid in the absence or presence

of glucose and/or ammonium sulphate. Both compounds repress urate oxidase synthesis and significantly influence the degree of proliferation of – enzymatically identical – microbodies (Table 1). In contrast to earlier observations on the turnover of microbodies in *H. polymorpha* (Veenhuis et al., 1983*a*), exposure of urate-grown cells of *T. cutaneum* and *C. famata* to glucose- or ammonium-excess conditions did not lead to degradation of the peroxisomes present in the cells. The observed inactivation of urate oxidase upon transfer of cells to glucose (Fig. 3) is therefore most probably due to a change in the physical state of the protein and may thus be considered a case of modification inactivation (Switzer, 1977).

As in other yeasts also the microbodies in *C. famata* and *T. cutaneum* are frequently observed in close association with strands of endoplasmic reticulum. The significance of these associations is still unclear. Most probably – as was indicated by our ultrastructural results – the endoplasmic reticulum is not involved in the biogenesis of the microbodies according to the classical model (De Duve and Baudhuin, 1966). In contrast, it must be expected that in yeasts urate oxidase is synthesized on free polysomes, similarly as in rat liver (Goldman and Blobel, 1978). However, the endoplasmic reticulum may be involved in the synthesis of the peroxisomal membrane. In this respect it must be stressed that, for reasons similar to those outlined for the other well-known examples of microbody proliferation in yeasts, also urate-grown *C. famata* and *T. cutaneum* are excellent candidates for the study of various aspects of microbody biogenesis.

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